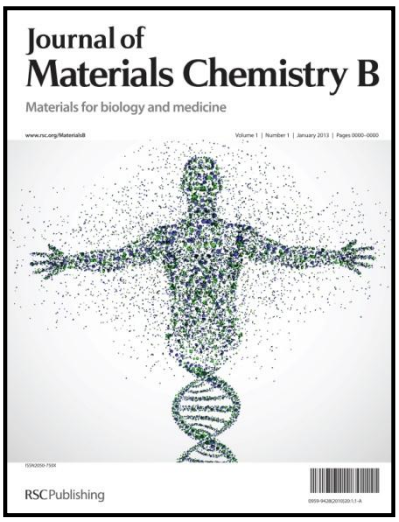
DOI:
10.1039/c3tb21604g

Document Version
Early version, also known as pre-print

Journal of Materials Chemistry B

The role of material structure and mechanical properties in cell-matrix interactions

Journal:	*Journal of Materials Chemistry B*
Manuscript ID:	TB-HIG-11-2013-021604.R1
Article Type:	Highlight
Date Submitted by the Author:	n/a
Complete List of Authors:	Evans, Nicholas; University of Southampton, Stem Cells and Regeneration, Bioengineering Sciences Group Gentleman, Eileen; King's College London, Craniofacial Development and Stem Cell Biology



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The role of material structure and mechanical properties in cell-matrix interactions

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Abstract

Cellular interactions with the extracellular matrix (ECM) are of fundamental importance in many normal and pathological biological processes, including development, cancer, and tissue homeostasis, healing and regeneration. Over the past few years, the mechanisms by which cells respond to the mechanical characteristics of the ECM has come under increased scrutiny from many research groups. Such research often involves placing cells on materials with tuneable stiffnesses, including synthetic polymers and natural proteins, or culturing cells on bendable micropost arrays. These techniques are often aimed at defining empirically the stiffnesses that cells experience in their interactions with the ECM, and measuring phenotypically how cells and tissues respond. In this review, we will summarise the evolution of materials for investigating cell and tissue mechanobiology. We then will discuss how material properties such as elastic modulus may be interpreted, particularly with regard to analytic measurements as an approximation of how cells themselves sense elastic modulus. Finally we will discuss how factors such as interfacial chemistry, ligand spacing, substrate thickness, elasticity and viscoelasticity affect mechanosensing in cells.

Introduction

Cells must interact with their environment in order to feed, grow and divide. To achieve this they must be able to sense properties of their external environment and respond accordingly. For example, for a simple single-celled organism like an amoeba to survive, it must be able to detect its prey, crawl towards it and engulf it. We know that these processes are carried out chemotactically – the cell detects chemicals in its surroundings and then 'follows its nose'. A great deal is known about the molecular mechanisms that control such processes in cells and multicellular organisms. But cell movement also depends on the ability of cells to crawl over or through a solid medium. This requires the cell to interact mechanically with its environment – the cell must be able to 'feel' the extracellular material it travels over, exert force on it, and move accordingly. In short, the cell must be mechanosensitive.

This is also true of the cells of multicellular organisms. During development and growth, cells are stretched, sheared and compressed, as indeed they stretch, shear and compress each other. And when we move, cells - particularly those in tissues like the musculoskeletal system - are subject to mechanical force. Cells are able to sense and respond to such applied forces, and indeed some have evolved as specialised mechanosensitive cells. This is illustrated elegantly by the hair cells of the mammalian inner ear. Hair cells are deflected by the vibrations caused by sound or acceleration and transmit signals to the brain that are interpreted as sound or movement[1].

But animal cells do not only respond passively to applied force, they must also feel the mechanical properties of the materials they inhabit by *applying* force to them. Like the amoeba, many animal cells interact mechanically with their neighbouring cells and with their surrounding extracellular matrix (ECM) to achieve a variety of physiological functions, including patterning tissues during development, enabling damaged tissues to heal, fighting infection, or growing and differentiating. To perform their correct physiological function, they must sense and understand the mechanical context of the material in which they reside. An increasing body of evidence now demonstrates that such processes are, in part, regulated by the mechanical equilibrium of their microenvironment, and in particular by the *stiffness* of their surroundings.

Stiffness is a general concept that describes the rigidity of a material, or how much it resists deformation in response to an applied force. Just as steel is stiffer than wood, bone is far stiffer than muscle, which is in turn stiffer than fat. To truly describe the inherent stiffness of a material, regardless of its size and shape, the more specific material property 'modulus' is often used. Modulus is a size-independent measure of a material's stiffness and is given in units of force per area, the equivalent of pressure. Biological tissues exhibit a wide range of values for modulus from hundreds of

pascals (Pa) for very soft tissues such as brain and fat, to tens of kPa for stiffer tissues such as muscle and up to MPa and GPa for bone[2]. Cells are remarkably versatile and can often be coaxed to grow on surfaces over a similarly wide range of stiffnesses, but designing and using materials to interpret a cell's response to stiffness, *per se*, is anything but simple.

History of ECM mechanosensing

Despite the recent research interest in mechanobiology, it had certainly been appreciated for some time that the mechanical properties of ECM could be detected and inform cells how to behave. Work during the 1970s and 1980s showed that different cell types, for example mammary epithelial cells, tended to proliferate on rigid surfaces, and differentiate on softer collagen gels[3]. Ingber and colleagues also recognised the relationship between the ability of a cell to exert force on an ECM, and its ability to spread and generate tension within its cytoskeleton[4] . In short, they hypothesised that matrix 'malleability' as they referred to it, affected the shape of a cell and therefore its behaviour. This was supported by other studies which examined the effect of cell shape alone. By varying the adhesiveness of tissue culture plastic, Folkman and Moscana showed that the degree to which a cell spread could be controlled, with a clear positive correlation between cell spreading and DNA synthesis[5]. These findings were extended by Watt *et al*. - by controlling the size of adhesive islands on which cells were allowed to spread, they demonstrated that cells from the skin epithelium divided less and differentiated more on small islands compared to large ones[6]. Very similar experiments were later conducted by Chen and colleagues. They found that by controlling the size of adhesive islands, constraints on cell spreading could promote apoptosis, an effect again ascribed to the cell's ability to make adhesions with the substrate and generate tension within its cytoskeleton[7].

Despite the now accepted fundamental requirement for cells to feel and respond to the stiffness of the material they grow on, robust experimental approaches were lacking until relatively recently. The first study to determine unambiguously the effect of ECM stiffness on cells was published in 1997 by Pelham and Wang[8]. The authors devised a method of creating elastic cell culture substrates with tuneable stiffnesses using the familiar laboratory material, polyacrylamide. Commonly used as a medium for the electrophoretic separation of proteins, they were able to adapt this material for cell culture studies by chemically cross-linking ECM proteins to its surface to promote cell attachment. Most importantly, they were able to vary the stiffness of the polymer by adjusting the concentrations and ratios of cross-linker to monomer, while chemically cross-linking the polymer to an underlying hard substrate for ease of manipulation.

Polyacrylamide has since been utilised in numerous studies to show how cell behaviour depends on substrate stiffness. In general, reduced cell spreading and increased motility have been reported on

softer, less stiff substrates (Figure 1). And on stiffer substrates, cells exert larger traction forces, create more stable focal adhesions and form more defined actin stress fibers (reviewed in[9]). Substrate stiffness was later shown to regulate cell movement[10] and cell differentiation and phenotype[11, 12]. Modifiable substrate systems were also used as a means of observing strain fields and calculating the traction forces exerted on substrates (now termed 'traction force microscopy') by various cell types[13, 14]. The importance of ECM mechanics as a fundamental mediator of cell behaviour reached a much wider audience with the publication of a review in *Science*[9] and a research paper in *Cell*[15] in 2005 and 2006, respectively. In the latter, Engler *et al*. utilised Pelham and Wang's polyacrylamide system to demonstrate that substrate stiffness alone could stimulate the differentiation of a putative source of stem cells – bone marrow stromal cells – to cell types as diverse as neurons, myocytes and osteoblasts. As a result of the success of the approach, the field has expanded considerably with a near-exponential increase in published papers and citations over the past 15 years (Figure 2).

The phenomenon of stiffness sensing is now recognised as being an interesting and important factor in a range of biological contexts. Discher's group had initially suggested that tissue stiffness could be an important factor in tissue repair: changes in the mechanical properties of tissues like muscle post-injury might subsequently, through mechanical mechanisms, cause pathological changes in the phenotype of cells within the tissues[12]. In addition, the finding of a tissue-dependent differentiation of putative stem cell populations from the bone marrow, combined with the prevailing view of them as circulating, engrafting stem cells, suggested they could contribute to tissue homeostasis and repair by sensing their engraftment site by mechanical as well as chemical means[15]. Such ideas have caught the imagination of researchers in the fields of regenerative medicine, tissue engineering and biomaterials, where the interaction of cells with biomaterials or with the tissues of a recipient is important to proper functioning of tissues during repair and regenerative processes[16]. For example, it is now becoming accepted that biomaterials used for implantation should recapitulate the mechanical characteristics of the tissue they are intended to replace[17]. As we have already seen, the stiffness-dependent differentiation of MSCs to different cell types supports this notion[15], and others have shown that earlier differentiation events, like those that occur during development, are also in part controlled by stiffness. For example, embryonic stem cells, or some of their early differentiation derivatives, are sensitive to substrate stiffness and make decisions to differentiate into cells of the three germ layers (precursors of adult cells comprising different regions of the body) depending on the stiffness of their growth environment[18].

However, changes in tissue stiffness may negatively impact on the healing of a tissue. For example, cardiomyocytes require an optimum stiffness in order to differentiate to functional myotubes[12] while increased stiffnesses, like those that might be found in post-infarction scar-tissue, lead to pathological function[19, 20]. This is also likely to be true of other tissues, for example endothelial cells subjected to a

stiff growth environment tend to lose contact with one another, a process that might be detrimental to proper function *in vivo*, where vessel integrity is key[21]. Cells derived from the liver too behave in a way that might result in pathological function *in vivo* when subjected to a stiff environments, expressing many of the features of scar-forming fibroblasts under stiff growth conditions[22, 23]. Similar mechanobiology-based ideas have been developed by groups working on cancer. Cancers often have different mechanical characteristics from their tissue of origin, and mechanical effects have been shown to be correlated with cancer metastasis. For example, mammographic density, an indicator of breast tissue stiffness, increases breast cancer risk[24], and may be related to tumour progression, metastasis and more aggressive cancers[25]. And indeed, it has been shown that matrix stiffness can influence the migration[26] and proliferation[27] of cancer cells.

Despite the many advances in our understanding of the biological context of material stiffness on cells, there is often scant attention paid to chemical and mechanical properties of the many materials used in such studies. Indeed, a number of reportedly stiffness tuneable cell culture systems are now regularly employed as standard practice with very little chemical analysis or materials characterisation carried out to verify their suitability for truly isolating their purported properties. Here, we take a renewed look at materials with tuneable stiffnesses for examining cell behaviour in 2D and attempt to understand how interfacial chemistry, ligand spacing, substrate thickness and measurements of elasticity and viscoelasticity affect interpretation of cell behaviour on these surfaces.

Mechanics of materials of cell culture substrates

Fundamentally addressing questions of how matrix stiffness affects cell behaviour requires using materials with reproducible, precisely defined stiffnesses. The engineering terms ‘Young’s’ or ‘elastic’ modulus are often employed to describe the size-independent stiffness of the flexible substrates used to culture cells. In its most simple form, elastic modulus describes the ratio between the force per unit area (stress) required to deform a material and the resulting fractional change in its length (strain). According to Hooke’s Law, if stress is plotted as a function of strain, the slope of the resulting curve will yield the elastic modulus. Elastic modulus calculated in this manner, however, relies on certain assumptions about the material – namely that it behaves as a continuum, is homogenous (isotropy), undergoes small deformations, and is linearly elastic (Figure 3). For classic engineering materials, including metals and most crystalline materials, these assumptions hold. However, for almost all biological tissues and many tuneable matrices used to study cell behaviour, they often do not, which complicates simple comparisons between materials.

When considering tuneable matrices utilised to study cell behaviour, some of the assumptions applied to calculate modulus are considered appropriate whilst others are not. No material is truly a

continuum. Matter itself is made up of discrete atoms, and many materials have nano, micro and meso-level features, but for most materials these characteristics are often ignored. The assumption of isotropy, however, is more difficult to dismiss depending on the system. Biological tissue is often hierarchically organised and it is almost never homogenous. Soft tissues such as skin have a preferred orientation of collagen and elastic fibers and this varies depending on location in the body. Similarly, bone is well known to be orthotropic or have different mechanical properties depending on its orientation when tested[28]. Polymer hydrogels and elastomers, however, can be formed with no true defined orientation and so should theoretically be identical in all directions and thus isotropic.

More difficult to dismiss is the assumption of 'small deformations', or that changes in geometry resulting from applied loads are negligible. For most engineering materials, 'small deformations' usually means less than 1% strain, and often less than 0.1%. Similar measurements to identify the mechanical properties of biological tissues often require more than 10% strain for tissues like ligament and more than 30% for blood vessels. Pelham and Wang's original measurements of polyacrylamide modulus were conducted under similarly large strain conditions, approaching as much as 100%[8]. As a result, the Hooke's law-based calculations of modulus they utilised are likely inaccurate even though their general conclusions still hold. Similarly, cells cultured within or on soft matrices can produce macroscopic deformations. Cells encapsulated within collagen hydrogels can contract the material to a fraction of their original size[29], and cells on 2D surfaces apply strains at their periphery in the range of 15 - 25%[10]. In these cases the resulting changes in the geometry of the material are difficult to neglect and more complicated formulations for calculating modulus that take into account large strains are usually required[30].

The assumption of linearity requires that the relationship between stress and strain be linear, or more simply, that doubling stress will double strain and *vice versa*. Some hydrogels, notably polyacrylamide, are considered to behave linearly (reviewed in[31]), however, most biological materials and many polymers do not. Collagen, which is the primary constituent of many soft tissues, possesses a distinctive 'crimp' pattern, which unfolds before the fibres themselves bear significant load. In general, the result is non-linear behaviour or high deformation under relatively low force when the tissue is first loaded, but increasingly higher forces as deformation increases, a phenomenon termed 'strain stiffening'. This property was thought to evolve as a means to protect tissues from damage due to large stresses, as tissues tend to become stiffer the more they are strained. Elasticity, on the other hand, requires that the material return to its original shape upon unloading and unload along the same path that it was loaded along, without a loss of energy. Polyacrylamide hydrogels display elastic behaviour, however, the elastomer polydimethylsiloxane (PDMS – discussed below), which is also regularly used to study the behaviour of cells on tuneable substrates, does not. PDMS, like many biological tissues, is instead viscoelastic or possesses time-dependent properties. Viscoelastic

materials continue to deform when left under an applied load over time (creep) or exert less stress over time when left under a constant strain (stress relaxation).

Moreover, the method used to determine the mechanical properties, usually modulus, of a tissue or tuneable cell culture matrix can have an extraordinary effect on the actual number reported. For example, measurements of the modulus of the human cornea range from less than 3 kPa to more than 19 MPa, depending on whether the tissue was tested by atomic force microscopy (AFM), in tension or with other testing modalities (reviewed in[32]). Similarly, SYLGARD 184, which is used to form PDMS, yields widely different elastic moduli depending on the testing regime. Materials formed from identical cross-linker:base component ratios are reported to have values for modulus that vary by up to three orders of magnitude when tested by nanoindentation (0.1 kPa)[33] as opposed to AFM (41 kPa)[18]. Although a full discussion of mechanical testing methods are beyond the scope of this review, Engler and colleagues have published on the importance of testing such substrates by AFM in order to effectively probe the mechanical properties at the scale of the cell[34]. Nevertheless, a standard measurement scheme is anything but widespread across the field.

Considered on the whole, strict comparisons of modulus among tuneable substrates for cell culture should not be conducted lightly. Different testing modalities can have large effects on the values obtained and when materials do not conform to the assumptions detailed above, Hooke's law may not be applicable, and defining the modulus of the material is far more difficult or even inappropriate.

Tuneable materials for determining cell response

Synthetic polymers

As mentioned above, the importance of substrate stiffness in regulating cell behaviour was first definitively demonstrated in cell culture experiments on polyacrylamide. Polyacrylamide is a highly water absorbent polymer formed from acrylamide subunits. Under aqueous conditions it acts as a hydrogel, or simply a water swollen network of cross-linked polymer chains. For cell culture studies, it is often formed via free radical polymerisation of acrylamide with the comonomer cross-linker bis-acrylamide. The resulting material is non-degradable, transparent, stable, and fouling-resistant. By varying the ratio of acrylamide to bis-acrylamide, it is possible to form hydrogels with elastic moduli in the range of 200 Pa to 40 kPa[34], although some groups report values higher than 700 kPa[33]. As previously noted, polyacrylamide is generally considered to behave as a linearly elastic material. However, as it is a fluid-saturated porous solid, polyacrylamide is probably best described as poroelastic rather than purely linearly elastic[35]. That is, polyacrylamide displays some time-dependent behaviour, but this results from fluid moving through the pores of the elastic solid rather than due to time-dependent flow of the material itself. Polyacrylamide is not permissive for cell attachment.

Therefore, to control ligand presentation and allow for cell adhesion, ECM molecules, often type I collagen, are covalently linked to the surface via a linkage such as with sulfo-SANPAH, an amide-reactive, light-activatable cross-linker based on aryl azide chemistry.

Another widely used polymer for analysing the effects of substrate stiffness on cells is polydimethylsiloxane (PDMS), a form of silicone, which is hydrophobic, and similarly to polyacrylamide, non-permissive for cell attachment. Most investigators form PDMS from a silicone elastomer kit, such as that available from Dow Corning, which forms a stable, transparent material upon mixing the base component with the curing agent[18, 33, 36]. Mechanical properties of the material can be altered by modifying the ratios of its constituents. Authors have reported PDMS stiffnesses ranging from 0.1 kPa to over 2 MPa[18, 33, 36]. As with polyacrylamide, cell attachment to PDMS requires functionalisation with ECM proteins. Unlike polyacrylamide, however, which is a hydrogel, PDMS is an elastomer, and although it can absorb some water it is not hydrated like a hydrogel. PDMS, like many polymers, is also well-recognised to be viscoelastic. Indeed, Trappmann *et al*. have reported that very soft PDMS surfaces are highly viscoelastic[33]. After a controlled indentation, the material was observed to nearly completely relax over a matter of minutes. Although PDMS has been used far less than polyacrylamide as a cell culture substrate, it is interesting to note that some of the pioneering experiments on traction forces used thin films of PDMS. In these experiments, a thin (1 μm) layer of the elastomer was formed on an underlying viscous layer. Cell tractions were observed by the cell-mediated wrinkling of the PDMS[37]. While this was a convenient technique for obtaining qualitative data on the forces cells exert on their substrata[38], traction force microscopy using polyacrylamide[13] has provided a simpler method for precisely measuring tractions and inferring forces and PDMS has resultantly fallen out of favour.

Although often used in 3D cell culture systems, which are beyond the scope of this review, poly(ethylene glycol) (PEG) can also be formed with different stiffnesses and utilised to exam cell behaviour in 2D systems[39]. PEG is a hydrophilic, water-soluble, non-immunogenic polymer approved by the FDA for human consumption[40]. When the terminal hydroxyl groups of PEG are substituted with acrylate groups to form PEGDA, the polymer can be cross-linked, usually by photopolymerisation, to form hydrogels. The stiffness of PEGDA hydrogels can be modified by varying either the molecular weight or the concentration of polymer in the gel. Resulting hydrogels have been reported with elastic moduli in the range of tens of kPa[41], similar to those reported for polyacrylamide. Like polyacrylamide, PEGDA hydrogels are highly resistant to protein adsorption and therefore require the binding of adhesive ligands to permit cell attachment. Nemir *et al.*, for example, have coupled the fibronectin-binding peptide sequence arginine – glycine – aspartic acid (RGD) to PEG-based hydrogels to allow for cell attachment[39].

Biologically derived materials

Hydrogels with varying stiffnesses can also be formed from a number of ECM proteins including collagen and fibrin, and from polysaccharides such as alginate and hyaluronan. Collagen hydrogels have been widely used for cell attachment and encapsulation for over thirty years[42] and are often used to evaluate the behaviour of cells under less defined conditions compared to experiments with synthetic materials – for example, comparing cell behaviour on relatively soft or stiff surfaces[43]. Acidified collagen can be formed into a stable hydrogel by a neutralisation reaction, which creates a surface for cell attachment or cells can be encapsulated within the material[29, 44]. The mechanical properties of protein-based hydrogels can be modified quite simply by changing the concentration of collagen (*e.g.*) within the gel. The plateau modulus (a more appropriate description for materials that display viscoelastic behaviour) of the resulting hydrogel will be approximately correlated with the square of the protein concentration[45]. Others have shown that the modulus of collagen hydrogels can be varied by compressing the gels[46], essentially forcing water out and increasing the concentration of protein. Modifying the hydrogel's mechanical properties by these methods, however, simultaneously alters the number of ligands available for cell attachment. Since it is well known that ligand presentation also affects cell behaviour[47], particularly on matrices stiff enough to allow for cell spreading[48], use of these systems makes decoupling the independent effects of each quite difficult. Complicating matters further, protein hydrogel systems such as collagen are viscoelastic and display nonlinear behaviour[49], *i.e.* like many biological tissues, they are 'strain stiffening'[50]. Whilst this is not likely a major concern for cells from very soft tissues, fibroblasts and mesenchymal stem cells, which can exert much larger traction forces, could presumably strain a substrate to such an extent that it becomes significantly stiffer, which can render interpretation of stiffness-based cell behaviour quite complicated.

Stiffness tuneable systems derived from polysaccharides such as alginate, a major component of brown algae, are also widely used in cell culture experiments. Alginate hydrogels can be formed by cross-linking their co-polymer blocks with divalent cations, often calcium[51], forming a hydrogel. Mechanical properties of alginate hydrogels can be manipulated by changing the concentration of alginate or the ionic cross-linker[52]. Because of alginate's simple cross-linking mechanism, however, it is unstable under normal cell culture conditions and its stiffness inevitably varies with diffusion of the cross-linking cations. Alginate, however, differs from collagen as like the synthetic polymers, it contains no native cell-binding ligands and so does not allow for cell attachment. Therefore, changing the stiffness of the material does not necessarily directly affect ligand presentation. In order to culture cells on its surface, the tethering of adhesive groups such as RGD are required[53].

Hyaluronan, a glycosaminoglycan abundant in many mammalian tissues, can also be formed into hydrogels upon chemical modification and can be tuned to provide surfaces with varying stiffnesses.

Rehfeldt *et al*. have demonstrated that cells cultured on hydrogel surfaces formed by cross-linking thiol-modified hyaluronic acid with PEGDA behave identically to those cultured on polyacryalmide surfaces (both functionalised with collagen), suggesting that the stiffness of the underlying matrix drives cell response rather than the chemical nature of the scaffold[54]. Young and Engler have since added a further element of control to such systems, by using the same materials to construct a hydrogel which stiffened gradually with time, recapitulating the natural stiffening seen in some developmental events, including cardiogenesis[55]. Here, the authors were able to control the time-dependent degree of gel stiffening by thiolating the hyaluronic acid with different concentrations of dithiothreitol. Gels were found to stiffen by a factor of between 2 and 3 during a period of approximately 100 hours, partially recapitulating the stiffening of the heart measured in embryonic chicks.

Issues with interpreting stiffness-mediated cell behaviour

One of the inherent problems with discerning the effects of substrate stiffness on cell behaviour utilising tuneable materials (whether they be synthetic or biologically derived) is that altering stiffness requires chemically modifying the material in some way. For most systems this involves changing cross-linking density, polymer molecular weight or concentration, which may concomitantly alter other factors, such as mesh size and surface chemistry. Altering these factors then potentially alters the binding of adhesive ligands. Because of these changes, it is often difficult to definitively discern whether cells sense substrate stiffness, the stiffness of the material between adhesion sites, or an alternative effect related to receptor-ligand binding characteristics on the altered surface.

This issue was addressed by Trappmann *et al*. who described how changes in mesh (pore) size in polyacrylamide hydrogels, rather than stiffness *per se*, regulated ECM tethering and thus epidermal and mesenchymal stem cell differentiation[33]. They describe how on very stiff polyacrylamide hydrogels, which are composed of tight polymer meshes, ECM tethering points are relatively close together compared on softer gels with their characteristic looser networks. By simple beam theory, displacement of a tethered molecule between two anchoring points will be a function of the distance between the tethering points cubed. In short, the strength of the feedback a cell experiences when applying a given load to a covalently linked ECM molecule will rapidly decrease with increasing distance between tethering points (Figure 4). They confirmed this hypothesis by culturing cells on soft hydrogels that had been artificially stiffened and on surfaces decorated with precisely spaced gold nanoparticles, which mimicked the spacing of the hydrogel meshes.

In contrast, Engler *et al.* had previously shown that the adhesion and spreading of rat aorta smooth muscle cells on polyacrylamide gels was insensitive to adhesive ligand density[48]. That is, on soft

hydrogels (E = 1 kPa), a wide range of collagen densities from 50 – 5000 ng/cm^2, failed to influence cell spreading. Instead, the stiffness of the matrix was the over-riding factor in determining cell shape. Supporting this, several studies have shown that cells are sensitive to the thickness of an elastic hydrogel chemically bound to an underlying stiff substrate (polyacrylamide gels, the most often-used hydrogel substratum for examining the effects of stiffness on cells, are almost always fabricated by covalently attaching the basal portion of the gel to a glass substrate (a coverslide) for ease of handling.). That is, cells on thin gels with low elastic moduli behave as they would on gels with much greater elastic moduli[56, 57] because an equivalent degree of cell contraction on a thin gel would require the cell to exert a greater strain on the gel (and therefore force), compared to on a thick gel (explained in more detail below). Trappman *et al.*'s theory contradicts these observations as it would predict no thickness-dependent effects – ligand spacing is identical *regardless* of the thickness of the gel.

Indeed, the issue of how far or deep a cell can 'feel' around itself and therefore how thick a substrate must be for a cell to detect only its stiffness is one of particular concern. Formal physical descriptions of this phenomenon have been given[58, 59]. But it can also be understood in simplified fashion by visualising a cell exerting a shear force on the surface of a gel, which can be approximated as having a direction parallel to the surface of the gel (please refer to Figure 5 for a diagrammatic explanation). On adherence to the substrate, the cell establishes focal adhesions and tugs the underlying ECM radially towards its centre. In gels of large thicknesses, the lateral distance that the cell is able to displace the gel at its periphery (given by *l* in Figure 5) is insignificant compared to the depth of the gel. Therefore, the extension of the gel along an imaginary line connecting the focal adhesion at the periphery of the cell to the underlying point at the gel/glass boundary where the gel is adhered is minor. However, when the thickness of the gel is reduced, the cell - in pulling laterally on the surface - would have to create a much greater strain on the gel to contract it an equivalent lateral distance. Again, considering an imaginary line connecting the focal adhesion to the adherence point of the gel to the glass, a lateral displacement *l* equal to that seen for the thick gel results in a much higher strain (which is, of course, the percentage extension of line A' to B' depicted in Figure 5). Note that the strain resulting from a fixed lateral displacement increases in a non-linear fashion (according to a power law) with decreasing substrate thickness (Figure 5b). Of course, the idea that the cell *would be capable* of contracting the gel an equivalent distance on a thin gel compared to a thick gel is purely hypothetical – in reality, the forces required to exert such strains on thin gels become too great for the cell, and this is in essence why the cell will be able to generate tension within its cytoskeleton and spread – it 'feels' the thin gel to be stiffer than their intrinsic elastic modulus would suggest. In fact, one can argue that the stiffness of the thin gel, as 'measured' by the cell *is* greater than that of the thick gel – here the dimensions of the gel and the measurement method (the cell is measuring the shear modulus of the surface) play a part in the recovered stiffness 'measurement', whereas the

independently measured E of the polyacrylamide, by for instance AFM measurements, may remain the same in both contexts.

Experimental observations confirm these theoretical explanations[60]. Using a system analogous to the fairy tale 'The Princess and the Pea', in which the princess feels the hard pea under a stack of mattresses, Kuo *et al*. experimentally determined that cells begin to 'feel' the underlying substrate when polyacrylamide hydrogels are less than 15 μm in thickness[57]. Similarly, mesenchymal stem cells cultured on very soft polyacrylamide surfaces that normally do not promote cell spreading, increase their spread area as gel thickness decreases from 30 to 5 μm[56]. And, Sen *et al*. utilised a finite element analysis to estimate the strain field generated by human mesenchymal stem cells[61]. Their models determined that cells on linearly elastic substrates (which include polyacrylamide hydrogels) can feel other cells approximately 40 μm away, approximately the length of a single cell.

Such effects may be exaggerated in cohesive groups of cells, such as in epithelia. Here, tissue dimensions are likely to become very important in the tissue 'stiffness' that cells feel. As groups of cells exert much greater forces on gels than single cells, and are able to deform them to a greater degree, such cell groupings may collectively 'feel' significant distances into substrata. Using the same principles discussed above for single cells, the lateral distance a colony is able to contract a gel is likely to be much greater than a single cell, and hence colony cells may work together to 'feel the pea' at significantly greater depths than single cells (Figure 5c). Some evidence supports this. Trepat *et al*. have observed that colonies of cells are (phenotypically) insensitive to intrinsic substrate modulus, which they attribute to the transmission of cell-induced forces across larger distances than for single cells[62]. Similarly, Mertz *et al*.[63] have shown greater force generation in small colonies of keratinocytes, an effect predicted theoretically by Banerjee *et al*.[64]. Such evidence suggests that experimental approaches for determining the phenotypic response of cell groups to substrate modulus must take into account such depth-sensing effects by, for example, modulating substrate depth. And such effects may have profound implications for physiological processes such as patterning or wound healing, where groups of cells crawl over thick, layered, mechanically heterogeneous substrata.

In such physiological examples, isotropic linearly elastic materials such as polyacrylamide do not exist; most ECM is composed of fibrous proteins. On protein gels constructed *in vitro* from collagen or fibrin, by comparison, cells appear to be able to deform the hydrogel as far as five cell lengths away[31]. Indeed, the half-maximal spread area of protein hydrogel systems is some ten-fold larger than that reported for polyacrylamide, and cells can sense the stiffness of the underlying matrix across far greater distances[65]. These observations are well accepted, however, the mechanism by which a signal might be propagated over such relatively large distances is unclear as the known strain stiffening properties of these gels are not sufficient to explain this behaviour[66]. The implication of these

observations is that the required thickness to ensure the cell only feels the intended matrix stiffness is highly dependent on the material. In short, any substrate must be sufficiently thick to avoid thickness effects, and strain stiffening materials such as fibrin and collagen gels must be much thicker than linear elastic materials such as polyacrylamide to ensure the cells detect the stiffness of the material alone.

Also of note is a consideration of how a substrate's stiffness is transmitted as information to the cell. As previously noted, most tuneable materials used for cell culture require the attachment of ECM proteins to foster cell adhesion. In such systems, the cell then 'feels' the substrate stiffness through the tethering molecular and its linkage. The resulting stiffness is then a combination of the matrix stiffness, that of its linking molecule and the ECM protein, and will depend on how the molecule is tethered to the surface and how the cell determines stiffness. Indeed, it remains unclear whether a cell applies a constant deformation and then monitors the resulting stress or if rather applies a constant stress and then determines the resulting deformation. Furthermore, although often ignored, all the components of this linking system can affect the feedback the cell receives. Indeed, even chemical linker themselves used to tether adhesive molecules to non-permissive surfaces appears to be able to affect cell behaviour. Houseman and Mrksich showed that 3T3 fibroblasts attached and spread less on surfaces with identical ligand presentation but longer linker groups[67]. The effect of the mechanical properties and/or length of the adhesive molecule itself are often not considered either. The bending modulus of hydrated single collagen fibrils has been estimated in the range of 10 to 100 MPa[68] and measurements collagen's elastic modulus range from 30[69] to 500[70] MPa. Thus in theory, the stiffness of the tethered collagen and other ECM molecules are far higher than that of many hydrogels and should act as a rigid tether, however, we are not aware of any studies that have definitively demonstrated this. Similarly, the covalent linkage that bonds the hydrogel to the ECM molecule, usually sulfo-SANPAH, is generally assumed to not play a role, but studies to explicitly determine this are lacking.

Cell-adhesive, bendable micropost arrays

Despite the success and interest in hydrogel and elastomer systems for studying the effects of matrix stiffness on cell behaviour, these materials are not ideal. Therefore, other options for isolating the effects of matrix stiffness independent from ligand presentation have been developed. Elastomeric (PDMS) hexagonally spaced micropost arrays micromoulded from silicon masters and functionalised by microcontact printing have been developed[71-74]. The resulting arrays can regulate apparent substrate stiffness via their length whilst presenting identical surface geometry and chemistry (Figure 6). Assuming cell traction forces are applied perpendicular to the surface, rigidity is correlated to post length and forces can be calculated based on post bending[71]. Human mesenchymal stem cells cultured

on short pillars behave as though they 'feel' a stiff substrate and behave as cells do when grown on stiff surfaces created from modifiable hydrogels - they spread and differentiate to osteoblasts[8, 71]. In contrast, cells cultured on long, easily bendable pillars behave as if they 'feel' a relatively soft substrate and adopt more rounded morphologies and become adipocytes. In short, numerous studies conducted on micropost arrays suggest that cells behaviour is mediated directly by substrate stiffness (reviewed in[75]) and cell behaviour examined using these surfaces correlates well with that determined by experiments on tuneable polyacrylamide hydrogels.

However, as with continuous deformable substrates, calculations of micropost array stiffnesses rely on their own set of assumptions, which should be carefully considered when making comparisons to other materials. The standard beam theory that is applied to these systems to calculate traction forces assumes the arrays are formed of 'slender beams' (aspect ratio > 10) that undergo small deflections relative to the height of the posts, and that their materials properties are constant. These assumptions do not necessarily hold for standard micropost array systems, particularly for long posts, *i.e*. soft substrates, that deflect a considerable amount under cell traction forces. Because micropost arrays do not conform to these assumptions, Lin *et al*., for example, have estimated that cellular traction forces could be overestimated by more than 60%[76]. Moreover, the substrate on which microposts are attached also deflects with applied stress. Not taking this deflection into account can also lead to errors in estimations of traction forces of some 40%[77]. Furthermore, if posts are spread too widely apart, cell spreading and movement can be affected[75].

Pure technical limitations also limit the more widespread use of micropost arrays. For example, it is not possible to form micropost arrays with effective elastic moduli below approximately 1.5 kPa[71]. Authors have reported particular cell behaviours below this stiffness range, including neuronal differentiation of human mesenchymal stem cells[15]. Furthermore, studies with polyacrylamide hydrogels show that cells deform the matrix in the z direction, or perpendicular to the culture surface, in addition to in the x-y directions, or the plane of the cell[78]. Such traction forces are not taken into account in micropost array systems as the z component of a force vector will not induce post bending. Instead, the cell will feel the inherent stiffness of the PDMS beam, whose stiffness will be independent of beam length. Finally, in contrast to hydrogel systems, micropost arrays are unlikely to be suitable for probing the effect of substrate stiffness on cohesive groups of cells. Here, large contractile forces generated by cell sheets may require the colony *as a whole* to contract by a much more significant degree than a single cell (tens of microns or more, compared to several microns). To accommodate such contraction, posts must be capable of bending a significant degree in the x-y directions. However, microposts that satisfy this requirement will need to be long, and during contraction, their surfaces will orient obliquely, and move out of the original plane of the culture surface. Furthermore, the bending of posts at the periphery of a colony will create a gap, preventing

the cells from actively probing outwards as the colony contracts. Such tests will remain easier to perform in hydrogels where the dimensions of the substrate can be adjusted to accommodate the extra strain.

Conclusions

The importance of tissue stiffness in directing the behaviour of adherent cells has advanced a great deal in the past fifteen years. We now have a wide array of systems, materials, mathematical models and measurement techniques to probe how cells respond to their mechanical microenvironments. As we have discussed in this review, there remain limitations to these technologies and gaps in our understanding that make interpretation of the cell's response to the mechanical properties of ECM challenging. Combined with the location of this field at the boundaries of cell biology, materials chemistry, engineering sciences, mechanics and physics, it is desirable that such fundamental problems continue to be addressed by truly multidisciplinary teams of researchers.

Tissue engineering strategies aim to design replacement tissues and organs by building them from cells and materials. Here the interaction of cells with an artificial material is critical. To address such questions in applied science and biomedicine, future studies must seek to tackle how groups of cells, behaving dynamically in three dimensional environments, are influenced by and exert influence on the mechanical properties of biomaterials. Although technically challenging, such questions are now being addressed, albeit at the single cell level[79, 80]. Future experimental protocols, whilst allowing for analysis of how individual cells spread on such surfaces and within such matrices, must take into account the complexity of tissues, where cells not only adhere to an ECM, but also adhere to each other and behave collectively. By working together too, there is great hope that researchers in the fields of biology, engineering, chemistry and physics may address such challenges in the not-too-distant future.

Biographies

Dr Nicholas D Evans is a Lecturer in the Centre for Human Development, Stem Cells and Regeneration and the Bioengineering Sciences Group at the University of Southampton. Nick received his PhD from King's College London for work on fluorescence sensor for diabetes in 2004 before taking up a position as a Medical Council Career Development fellow in stem cell research at Imperial College London. After a brief postdoctoral position at Stanford University, USA, Nick took up his current position at Southampton in 2011. Nick now leads a multidisciplinary group researching methods to promote tissue regeneration in bone and skin.

Dr Eileen Gentleman is a Wellcome Trust Research Career Development Fellow in the Department of Craniofacial Development & Stem Cell Biology at King's College London. After completing her PhD in Biomedical Engineering at Tulane University (USA) in 2005, Eileen joined Imperial College London as a post-doctoral research associate. In 2011, she moved to King's where her research focuses on utilising biomaterial systems to direct stem cell differentiation to bone and cartilage. Her multi-disciplinary research group also studies biomineralisation and the role of mechano-sensing in tissue development. Her work at the engineering-biological science interface has recently been recognised with a prestigious Philip Leverhulme Prize (2013).

Figure Captions

Figure 1: Cells on stiff and soft surfaces. a) Cells (blue) cultured on stiff substrates cannot deform their matrix, adopt a spread morphology, and develop defined stress fibres. Inset shows differentiating mouse embryonic stem cells cultured on a polydimethylsiloxane surface with an elastic modulus of 2.7 MPa (as determined by atomic force microscopy, see Evans *et al*.[18] for a full description of methods). Phalloidin labelling of actin shows well-defined stress fibre formation. Scale bar = 100 μm. **b**) Traction forces generated by cells cultured on relatively soft substrates deform the underlying matrix. Cells appear rounded and lack pronounced stress fibres. Inset shows differentiating mouse embryonic stem cells cultured on a polydimethylsiloxane surface with an elastic modulus of 41 kPa Phalloidin labelling demonstrates diffuse actin fibre formation. Scale bar = 50 μm.

Figure 2: Cell mechanobiology publications. Database analysis (SCOPUS) of publications in the field of cell mechanobiology using the terms "substrate AND (elasticity OR stiffness) AND cells". **a**) The number of items published between 1997 and 2013. **b**) The number of citations. Both show nearly exponential growth in research interest in the field.

Figure 3: Hooke's law. a) The mechanical properties of a material with defined cross-sectional area (A) can be determined by applying a force (F) and measuring the fractional change in length (l). **b**) According to Hooke's Law, if stress (σ) is plotted as a function of strain (ε), the slope of the resulting curve will yield Young's or elastic modulus (E). As a result, stiffer tissues such as bone have a larger E than softer tissues such as cartilage and fat. **c**) Hooke's Law, however, is a simplified formulation and relies on a number of assumptions for calculations of E to be valid. Many biological tissues and materials used as tuneable substrates for cell culture do not meet the assumptions for Hooke's Law.

Figure 4: Cells sense extracellular matrix tethering separation. Trappman *et al*.[33] provided evidence to suggest that ligand spacing, rather than substrate stiffness, is responsible for the altered cell behaviour on polyacrylamide gels that vary in stiffness. By artificially stiffening gels of low elastic modulus (with less dense polymer networks; yellow, top panel) or high elastic modulus (with denser polymer networks; yellow, lower panel) they were able to still observe differences in parameters such as cell spreading, despite the equivalent high stiffnesses. They attribute this to a reduction in the density of binding sites (blue circles) of the type I collagen matrix coating (crimson lines) to the underlying hydrogel (yellow network), and a reduced ECM mechanical feedback, which declines as a function of the cube of the distance between tethering points. This is illustrated in the top panel as an increased bending of collagen fibrils during cell contraction (crimson dashed lines prior to cell contraction and solid crimson lines after contraction). The cell is illustrated in salmon pink with a

blue nucleus, and cytoskeletal elements are shown as purple lines, with increased tension shown as a darker colour.

Figure 5: Cells sense substrate thickness. a) The cartoon depicts in a simplified form the difference in strain that a cell must exert by contracting an equivalent amount on thick and thin gels of equal elastic modulus. Here the strain is measured as an extension in the gel on a line between a focal adhesion at the periphery of the cell and a point of adherence of the gel to the underlying glass at the position directly beneath the focal adhesion. After adhering to a thick gel and forming a focal adhesion, the cell exerts a contractile force on the gel (top left). If the gel deforms a distance *l* (top right), the strain in the stated direction ($\varepsilon(A\rightarrow B)_{thick}$) is given by (B-A)/A. For a thin gel, if the cell adheres to the gel forming a focal adhesion (bottom left) and deforms the gel an equivalent distance *l* the strain ($\varepsilon(A'\rightarrow B')_{thin}$), given by (B'-A')/A' is much greater than that for the thick gel. The stress required for the deformation in the latter case may be greater than that the cell is able to exert, and therefore the tension within the cell reaches a critical threshold and the cell spreads, whereas in the former case, the cell may be unable to generate the same degree of cytoskeletal tension and may remain rounded. Note that in both cases the elastic modulus of the material is the same. Note that this figure is for explanatory purposes only and ignores many other variables - for a full physical description of depth sensing please refer to Merkel *et al.*[58] or Maloney *et al.*[59]. **b**) The graph shows that the strain required to deform the gel a distance *l* increases according to a power law with decreasing gel thickness (A). The strain in the direction indicated is given by the percentage extension of the hypotenuse of an imaginary triangle with vertices marking (1) the focal adhesion at the gel surface prior to a hypothetical cell contraction (2) the focal adhesion at the gel surface after a hypothetical cell contraction and (3) the point of adherence of the gel to the underlying glass support directly below vertex (1). **c**) For cohesive cell layers, such as in colonies of cells, the distance *l* is likely to be greater than for a single cell. Therefore, it is possible that cells, acting collectively in colonies, detect depth-dependent increases in substrate stiffness at greater gel thicknesses than for single cells.

Figure 6: Micropost arrays. Micropost arrays are micromoulded from silicone elastomers and functionalised to permit cell attachment. Cartoons depictions of effectively **a**) relatively soft and **b**) stiff surfaces created from long and short posts, respectively. **c**) For a given force F_1, the 'soft' long pillar will bend more easily whilst the 'stiff' short pillar will bend far less.

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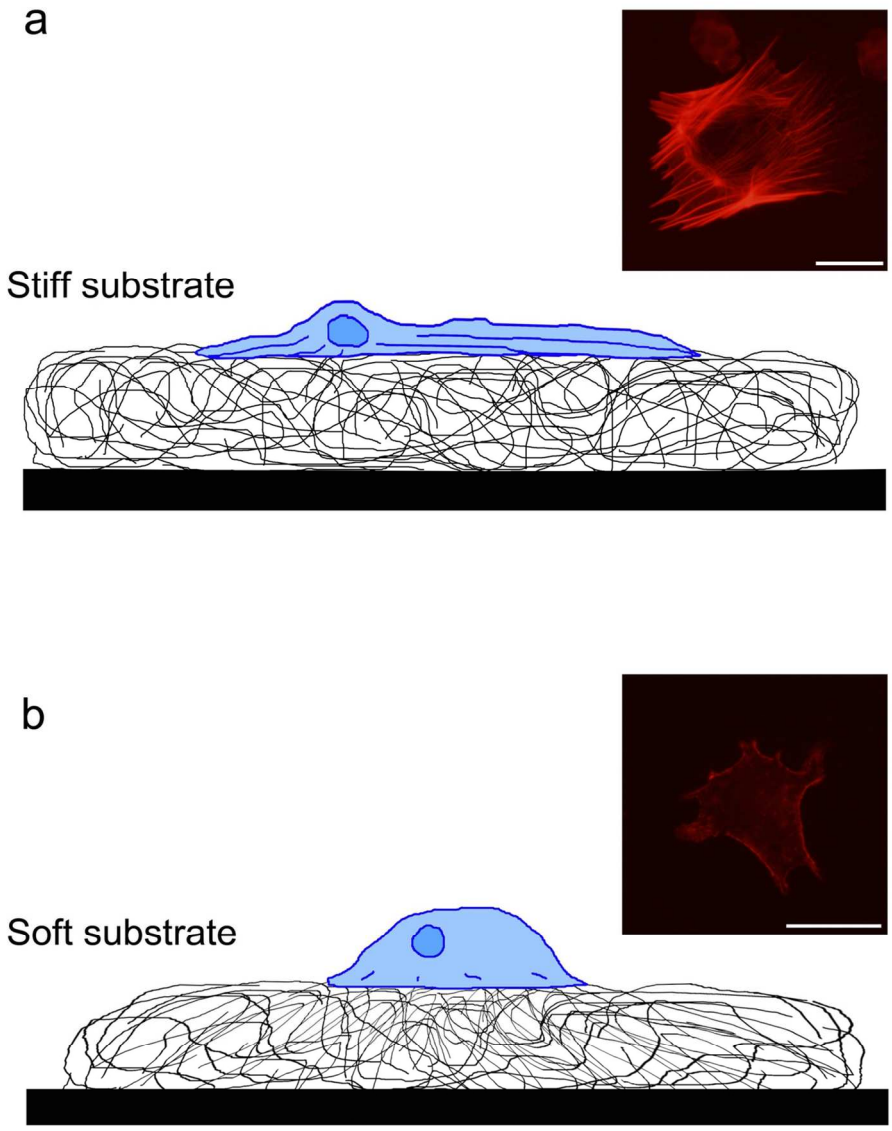

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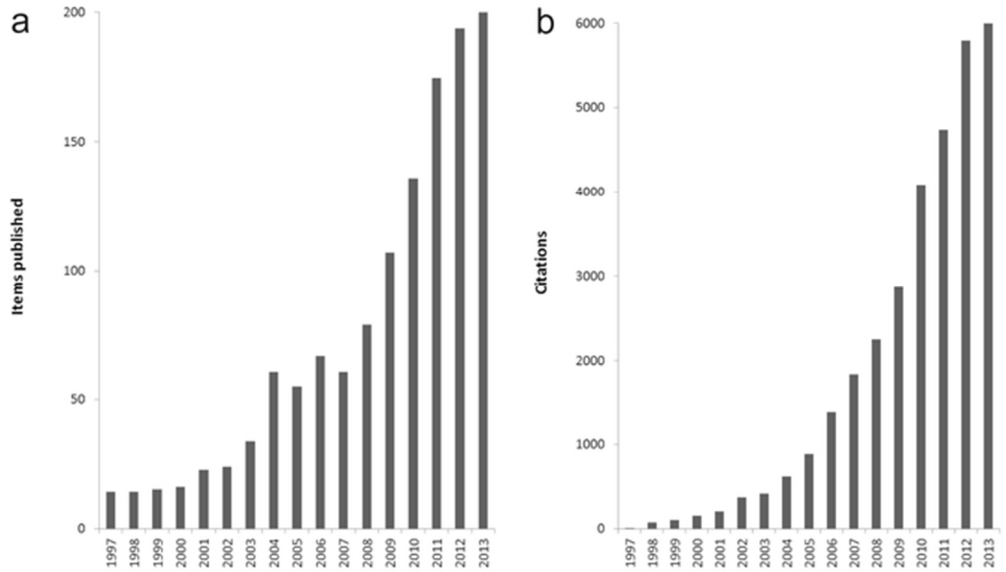

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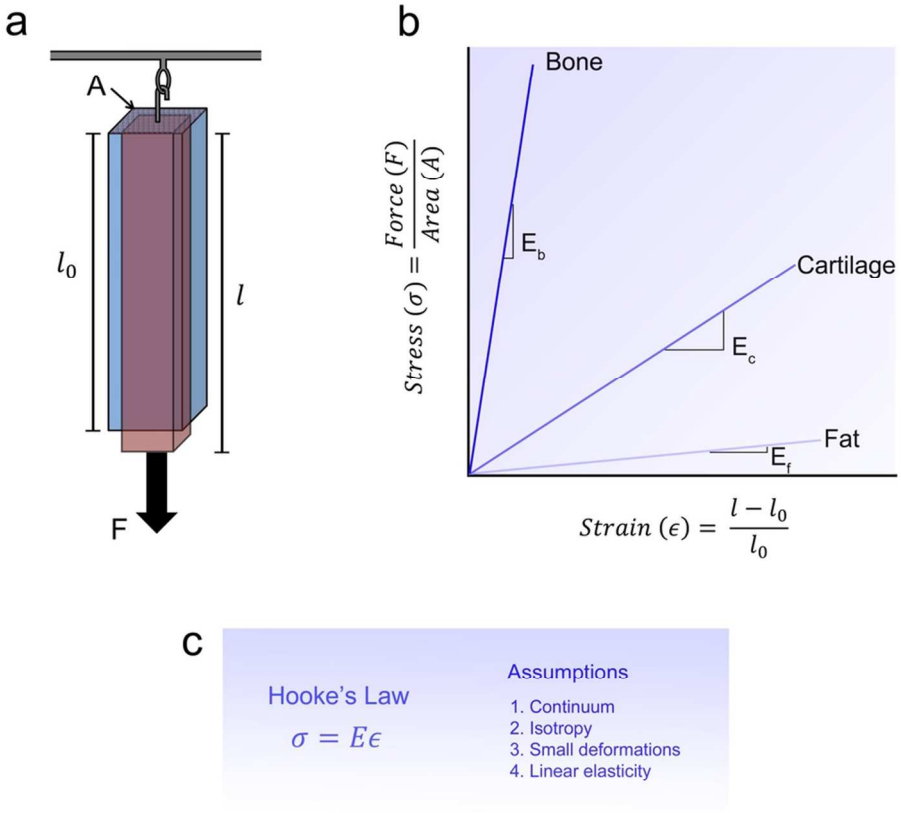

Figure 3: Hooke's law. a) The mechanical properties of a material with defined cross-sectional area (A) can be determined by applying a force (F) and measuring the fractional change in length (l). b) According to Hooke's Law, if stress (σ) is plotted as a function of strain (ε), the slope of the resulting curve will yield Young's or elastic modulus (E). As a result, stiffer tissues such as bone have a larger E than softer tissues such as cartilage and fat. c) Hooke's Law, however, is a simplified formulation and relies on a number of assumptions for calculations of E to be valid. Many biological tissues and materials used as tuneable substrates for cell culture do not meet the assumptions for Hooke's Law.
90x81mm (300 x 300 DPI)

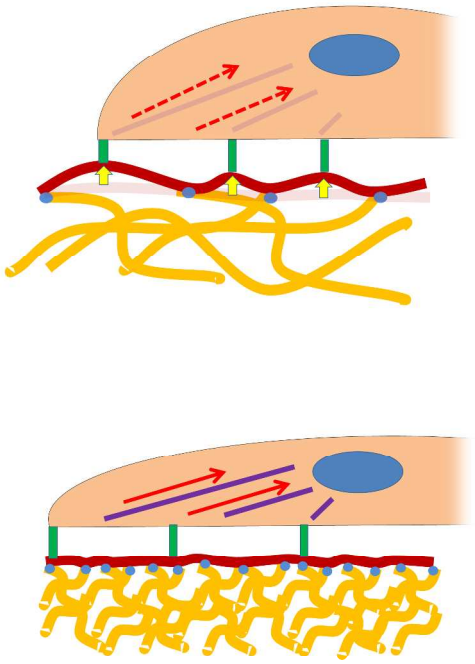

Figure 4: Cells sense extracellular matrix tethering separation. Trappman et al.33 provided evidence to suggest that ligand spacing, rather than substrate stiffness, is responsible for the altered cell behaviour on polyacrylamide gels that vary in stiffness. By artificially stiffening gels of low elastic modulus (with less dense polymer networks; yellow, top panel) or high elastic modulus (with denser polymer networks; yellow, lower panel) they were able to still observe differences in parameters such as cell spreading, despite the equivalent high stiffnesses. They attribute this to a reduction in the density of binding sites (blue circles) of the type I collagen matrix coating (crimson lines) to the underlying hydrogel (yellow network), and a reduced ECM mechanical feedback, which declines as a function of the cube of the distance between tethering points. This is illustrated in the top panel as an increased bending of collagen fibrils during cell contraction (crimson dashed lines prior to cell contraction and solid crimson lines after contraction). The cell is illustrated in salmon pink with a blue nucleus, and cytoskeletal elements are shown as purple lines, with increased tension shown as a darker colour.
1000x1000mm (78 x 78 DPI)

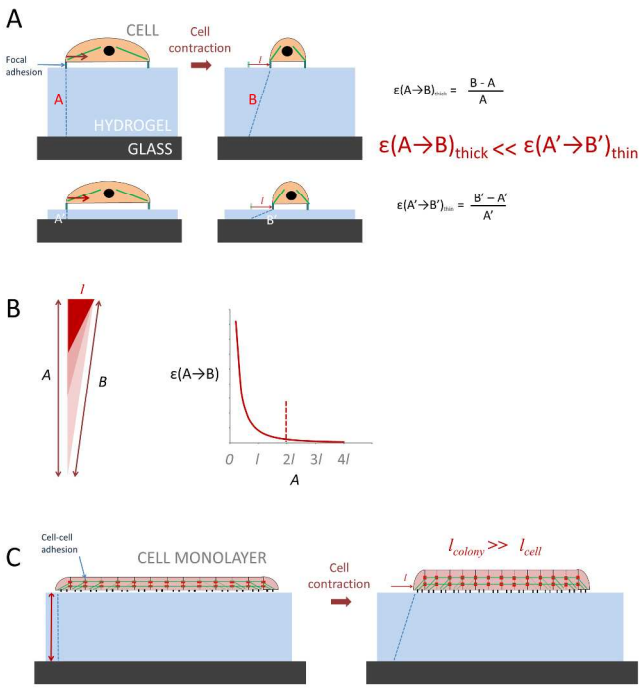

Figure 5: Cells sense substrate thickness. a) The cartoon depicts in a simplified form the difference in strain that a cell must exert by contracting an equivalent amount on thick and thin gels of equal elastic modulus. Here the strain is measured as an extension in the gel on a line between a focal adhesion at the periphery of the cell and a point of adherence of the gel to the underlying glass at the position directly beneath the focal adhesion. After adhering to a thick gel and forming a focal adhesion, the cell exerts a contractile force on the gel (top left). If the gel deforms a distance l (top right), the strain in the stated direction (ε(A→B)thick) is given by (B-A)/A. For a thin gel, if the cell adheres to the gel forming a focal adhesion (bottom left) and deforms the gel an equivalent distance l the strain (ε(A′→B′)thin), given by (B′-A′)/A′ is much greater than that for the thick gel. The stress required for the deformation in the latter case may be greater than that the cell is able to exert, and therefore the tension within the cell reaches a critical threshold and the cell spreads, whereas in the former case, the cell may be unable to generate the same degree of cytoskeletal tension and may remain rounded. Note that in both cases the elastic modulus of the material is the same. Note that this figure is for explanatory purposes only and ignores many other variables - for a full physical description of depth sensing please refer to Merkel et al.58 or Maloney et al.59. b) The graph shows that the strain required to deform the gel a distance l increases according to a power law with decreasing gel thickness (A). The strain in the direction indicated is given by the percentage extension of the hypotenuse of an imaginary triangle with vertices marking (1) the focal adhesion at the gel surface prior to a hypothetical cell contraction (2) the focal adhesion at the gel surface after a hypothetical cell contraction and (3) the point of adherence of the gel to the underlying glass support directly below vertex (1). c) For cohesive cell layers, such as in colonies of cells, the distance l is likely to be greater than for a single cell. Therefore, it is possible that cells, acting collectively in colonies, detect depth-dependent increases in substrate stiffness at

greater gel thicknesses than for single cells.

1000x1000mm (78 x 78 DPI)

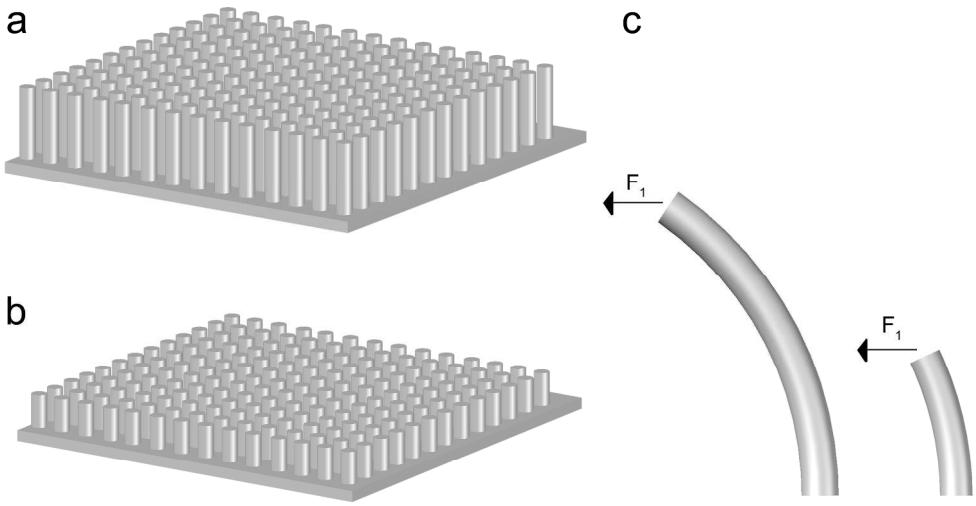

Figure 6: Micropost arrays. Micropost arrays are micromoulded from silicone elastomers and functionalised to permit cell attachment. Cartoons depictions of effectively a) relatively soft and b) stiff surfaces created from long and short posts, respectively. c) For a given force F1, the 'soft' long pillar will bend more easily whilst the 'stiff' short pillar will bend far less.
251x140mm (300 x 300 DPI)